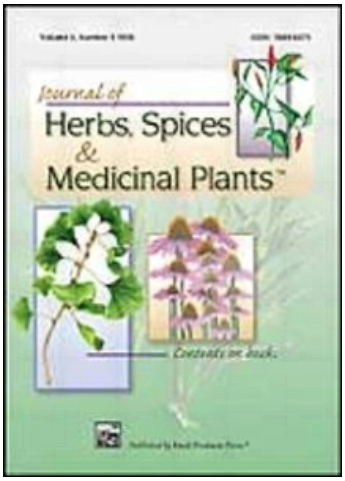

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Identification and Differentiation between *Hoodia gordonii* (Masson) Sweet ex Decne., *Opuntia ficus indica* (L.) P. Miller, and Related *Hoodia* Species Using Microscopy and PCR

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Hoodia gordonii*, an appetite suppressant, is used extensively as a botanical dietary supplement. Microscopy and molecular genetic procedures are provided for identification and differentiation between* H. gordonii, Opuntia ficus indica*, and other related* Hoodia *species.*

KEYWORDS ghaap medicinal plant, microscopic identification, molecular marker, weight loss

INTRODUCTION

The genus *Hoodia* (family Asclepiadaceae, previously Apocynaceae), which consists of approximately 13 species (1, 7, 11), is a succulent with a spiny appearance similar to cacti, although the plant is not a cactus. Commonly known as *ghaap* (6), *H. gordonii* (Masson) Sweet ex Decne is distributed from Brandberg in Namibia south into Cape Province, where the plant is scattered over much of the Great Karoo to the Tanqua Karoo in the southwest and the Prince Albert district in the extreme south (4). The San Bushman of the Kalahari Desert consumed the bitter tasting plant during their long

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hunting trips to stave off hunger and to sometimes use the juicy young shoots, raw or cooked, as food (11). P57 oxypregnane steroidal glycosides isolated from *H. gordonii* acts as an appetite suppressant (3, 8).

As a rare succulent, *H. gordonii* is listed by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) and is protected by national conservation laws in South Africa and Namibia. The plant can only be collected or grown with a permit (6). With the increase in popularity of *Hoodia* as an appetite suppressant in mid-2004 and a growing demand for weight-loss botanicals, the limited supply of *Hoodia* has created possibilities of substitutions and adulteration of the plant material (3). The American herbal Pharmacopeia that noted *Hoodia* is occasionally erroneously referred to in the media and online websites as a cactus.

The prickly pear cactus (*Opuntia ficus indica* (L.) P. Miller) is oftentimes confused with *H. gordonii*, but this cactus in commerce is not related to *Hoodia* and has no reported appetite-suppressant activity. Recent research has focused on chemical fingerprinting and presents a detailed analysis of *Hoodia* by high-performance thin-layer chromatography (HPTLC) (13) and high-performance liquid chromatography (HPLC) using UV detection (2). Yet, available pharmacopoeias lack macroscopic/microscopic authentication and identification based on DNA markers for *H. gordonii*.

In the present study, a detailed macroscopic and microscopic account of *H. gordonii* and a comparative account of related *Hoodia* species, such as *H. currorii* (Hook.) Decne., *H. parviflora* N.E Br., and *H. ruschii* Dinter, and the cacti *O. ficus indica* has been developed to identify contamination and substitutions for *H. gordonii*. In addition, dietary supplements claiming to contain *H. gordonii* plant material were microscopically analyzed and a molecular method to help identify *Hoodia* and distinguish the plant from *O. ficus indica* was developed. We isolated genomic DNA from 23 dried plant samples and analyzed two chloroplast genomic regions by polymerase chain reaction (PCR). The PCR products were cloned and sequenced. The sequence comparison revealed minor and major differences between the analyzed species. Based on these differences, primer pairs were designed in order to aid in the authentication and the presence of *Hoodia* spp. or *Opuntia* spp. DNA in powdered plant material.

MATERIALS AND METHODS

Plant Material

Authenticated voucher samples of *H. gordonii* were procured from Missouri Botanical Garden (MBG; St. Louis, MO; Voucher No. 2821) and the Universiteit Van Die Vrystaat, South Africa (Voucher Nos. 2925, 2926, 2927). Vouchers for *Hoodia currorii* (Voucher No. 2823) and *Hoodia ruschii* (Voucher No. 2822) were procured from MBG. Samples of *H. gordonii*

(Sample Nos. 2799, 2887, 3126, 3164, 3165, 3166) were procured from American Herbal Pharmacopeia® (AHP; Scotts Valley, CA) and Chromadex (Irvine, CA). Commercial products containing *Hoodia* (Sample Nos. 2869, 2870, 2871, 2873, 2874, 2875, 2876, 2877, 2878, 2879, 2889, 3167, 3168, 3169, 3171) were also analyzed. Samples of *H. currorii* (No. 3161), *H. ruschii* (No. 2952), and *H. parviflora* (Voucher No. 3162, 3163) were procured from AHP.

The vouchers for *O. ficus indica* (Voucher Nos. 2886 and 2888) were collected from different locations in Texas and identified by Dr. Charles Burant, University of Mississippi. Vouchers for the Asclepiadaceae were procured from MBG and included *Ceropegia dichotoma* (Voucher No. 2820), *Cynanchum perrieri* (Voucher No. 2824), *Cynanchum marnieranum* (Voucher No. 2825), *Edithcolea grandis* (Voucher No. 2815), *Huernia recondita* (Voucher No. 2818), *Huernia keniensis* var. *grandiflora* (Voucher No. 2819), *Orbea variegata* (Voucher No. 2816), *Orbea variegata* (Voucher No. 2817), *Paranthus globosus* (Voucher No. 2814), *Stapelia gigantea* (Voucher No. 2809), *Stapelia schinzii* (Voucher No. 2810), *Stapelia leendertziae* (Voucher No. 2811), *Stapelia flavirostris* (Voucher No. 2812), and *Tridentea choanantha* (Voucher No. 2813). Vouchers and samples for all the plants are deposited at the National Center for Natural Products Research Repository (University of Mississippi, MS).

Macroscopic and Microscopic Analysis

The collected *Hoodia* species, the *O. ficus indica* samples, and other succulents were freeze dried and then hand sectioned. Sections were treated with chloral hydrate as a clearing agent and then observed at different magnification under a Nikon Eclipse E 600 microscope (Nikon Instruments, Inc., Melville, NY) and images were recorded with a Kodak digital camera (Model DC290, Eastman Kodak Co., Rochester, NY) and processed using Adobe Photoshop® (Adobe Systems, Inc. San Jose, CA). Commercial dietary supplements with labels claiming to contain *H. gordonii* whole plant material were also analyzed microscopically. Tablets of the commercial samples were macerated in NaOH and nitric acid and then examined using the microscope described above.

DNA Extraction and PCR Reactions

DNA from Asclepiadaceae, Cactaceae, and commercial samples labeled 1, 2, 3, and 15 (Tables 1 and 2) were extracted using a modified cetyltrimethylammonium bromide (CTAB) method described by Wang et al. (12). To characterize the *atp*B-*rbc*L and *psb*A-*trn*H intergenic regions for the *Hoodia* and *Opuntia* samples, we PCR amplified the regions with Taq DNA polymerase

TABLE 1 Macroscopic and Microscopic Analysis of *Hoodia* and Related Species

Genus/character	*Hoodia gordonii* (Masson) Sweet ex Decne	*Hoodia currorii* (Hook.) Decne.	*Hoodia ruschii* Dinter	*Hoodia parviflora* N.E Br.
Macromorphology				
Stem diameter (cm)	2–5	4–6	1.5–1.8	3–3.5
No. of tubercules	11–17	17	22–28	11–12
Spines	Curved to straight ca. 0.6–1.2 cm long, grayish green to brown	Curved to straight, 0.6–1.5 cm long, grey to brownish green	ca. 0.4 cm long, stiff, fragile, brownish-grayish green	ca. 0.2 cm short, stout, violet green
Distance between epidermis to cortex (cm)	ca. 0.6	ca. 0.8	ca. 0.4	ca. 0.7
Pith diameter (cm)	ca. 1.5	ca. 2–2.5	ca. 1.3	ca. 2
Micromorphology				
Palisade cortical cells	ca. 100–300 μm long, outer cortical cells turgid, few cells containing chlorophyll, inner cortical cells slightly undulated	ca. 100–500 μm long, outer cortical cells turgid, partially filled with chlorophyll, presence of several laticiferous cells	ca. 70–100 μm long, cortical cells containing several starch grains	ca. 100–150 μm long, highly undulated/ collapsed/ shrunken, of laticiferous canals
Presence of intercellular spaces	Cortical cells with intercellular spaces rarely observed	Large intercellular space just below epidermis in outer cortical cells	Intercellular spaces not observed	Large intercellular spaces in inner cortex near vascular bundles and pith
Vessels	ca. 30–40 μm wide with multiple cells, scalariform pitting,	ca. 40–50 μm wide, with multiple cells with scalariform pitting	ca. 10–20 μm wide with scalariform pitting, multiple cells	ca. 30–100 μm with scalariform pitting, multiple cells

high fidelity and cloned and sequenced all the generated PCR products (Invitrogen Carlsbad, CA). The two intergenic regions *atp*B-*rbc*L and *psb*A-*trn*H were amplified from genomic DNA using the primer combinations *atp*B-1 (ACATCKARTACKGGACCAATAA), *rbc*L-1 (AACACCAGCTTTRAATC-CAA) (5), *psb*A-*trn*H F (GTTATGCATGAACGTAATGCTC), and *psb*A-*trn*H R (CGCGCATGGTGGATTCACAAATC) (9). The PCR reactions were done with 10 ng DNA as a template in 25-μL reactions as described by Techen et al. (10) and the reactions were purified, extracted, and cloned as described by Van Heerden (11). DNA from two to three colonies of each

TABLE 2 Showing Commercial Analyzed Samples

Commercial sample	Type	Presence of *Hoodia* Species*
2889	Capsules	+++
3168	Capsules	–
3167	Capsules	+++
2877	Capsule	–
2869	Tablet	Several fragments of fiber
2871	Tablet	+ Small fragments of vessels
2870	Tablet	–
2874	Tablet (white)	–
2879	Tablet (Red)	–
2977	Tablet (white)	+
2873	Tablet (red)	+
2875	Tablet (white)	–
2876	Tablet (white)	–
2978	Tablet (white)	–
3171	Tablet (white)	–

*+ = Present; – = absent or undetected *Hoodia*.

sample was isolated with the Spin Miniprep Kit (Catalog No. 27106, Qiagen Inc., Valencia, CA) and double-stranded sequenced on an automated DNA sequencer (Model ABI 3730XL, Applied Biosystems, Foster City, CA).

Based on the differences between the sequences, three forward primers and two reverse primers were designed: *psb*A-*trn*H intergenic region primer pair 1: F6_Hoo_psbA (AAAAATAAATTAAATAAAAAAAAAT-TATAATATTAAATAAAAAATAAAAA)/R3_Hoo_psbA (CTTTATTGCCTTTG-GTTAATTC); *psb*A-*trn*H intergenic region primer pair 2: F8_Hoo_psbA (TAAATAAAAAAAATTTTAATTTATTTTTTTTTATTTAATATATAAATATA)/R3_ Hoo_psbA; *atp*B-*rbc*L intergenic region primer pair 3: F1_Opu_atpB (GAAT-TCTCAAGTTCACTCAACTGATTT)/R1_Opu_atpB (TTTAAATATAGTTTCGA GTTCGACTTTC). Template for the nested PCR was 1 μL of a 1:10 dilution of the first PCR.

RESULTS AND DISCUSSION

Macroscopic Authentication

The stem, ca. 0.5 to 1 m tall, grayish green, cylindrical, straight, or slightly bending, ca. 2–5 cm diameter, tubercules arranged in 11–17 ridges; tubercules protruding, blunt with a sharp spine; spines 0.6–1.2 cm long, grayish green to brown, mostly facing down, slightly curbed. Taste: slightly sweet at first and later a lingering bitter.

Microscopic Authentication

Transverse section of *H. gordonii* shoot shows an outer epidermis, followed by palisade cortex ca 0.6 cm wide and an enlarged pith 1–1.5 cm in diameter (Fig. 1A); epidermis thin walled, tabular, parenchymatous, with stomata (Fig. 1B); epidermis followed by single or two- to three-celled, thin-walled hypodermis, cells rectangular; followed by palisade cortex, collenchymatous, cells up 300 μm long, outer turgid, thin walled, ovate to oblong, perpendicular to the stem surface, with few intercellular spaces, supported by numerous strands of fibers (Fig. 1C); circle of bicollateral vascular bundles supported by fibers; tracheary elements at the junction of inner cortex and an enlarged pith (Fig. 1D); vessels 6–8 in a cluster (Fig.e 1E), ca. 500–600 μm long × ca. 30–40 μm wide, scalariform pitting, numerous multiples of cells joint together (Figs. 1F, 1G); fiber simple, with horizontal striations (Fig. 1H); starch gain almost absent, rarely a few present near the palisade cortical cells surrounding the tracheary elements; oxalate crystals absent; pith with several arenchymatous cells and containing laticiferous canals and several supporting fibers.

O. ficus indica, of the family Cactaceae, can be easily differentiated from *H. gordonii*, by palisade cortical cells, ca. 200–300 μm long and 100 μm wide, containing distinct oxalate crystals (druses) mixed with mucilage cells (Figs. 2A, 2B). The plant contains canals of mucilaginous cells lying parallel to the vascular bundles and contains vessels with simple pits (Fig. 2D).

Oxalate crystals were absent in *H. gordonii* and other studied *Hoodia* species. In powder form, samples of *H. currorii*, *H. ruschii*, and *H. parviflora* have similar anatomical characteristics to *H. gordonii*, making identification of exact *Hoodia* species difficult, if not impossible. Macroscopically and microscopically, however, tissue samples of the four species can be differentiated (Table 1). *H. currorii* has much larger palisade cortical cells ca. 100–500 μm long and outer cortical cells with cholorphyllous content (Fig. 3B). *H. parviflora* has relatively small outer cortical cells, ca. 100–150 μm long, highly undulated, collapsed, and shrunk with several laticifers and distinct well developed vascular bundles in the inner cortex (Fig. 3C). Vessels in *H. parviflora* are alternating with several supporting fibers surrounding the vessels (Fig. 3C). Cortical cells of *H. ruschii* (ca. 70–100 μm) are relatively small compared to *H. gordonii* and other *Hoodia* species analyzed and have several starch grains with large intercellular spaces (Fig. 3D). The cortical cells, however, are not fully covered with starch grains. *H. currorii* showed the presence of some melanin in outer cortical cells along with presence of chlorophyll (Fig. 3B).

Dietary Supplement Analysis

Fifteen dietary supplements claiming the presence of *H. gordonii* plant material were analyzed to determine whether they contained *H. gordonii*

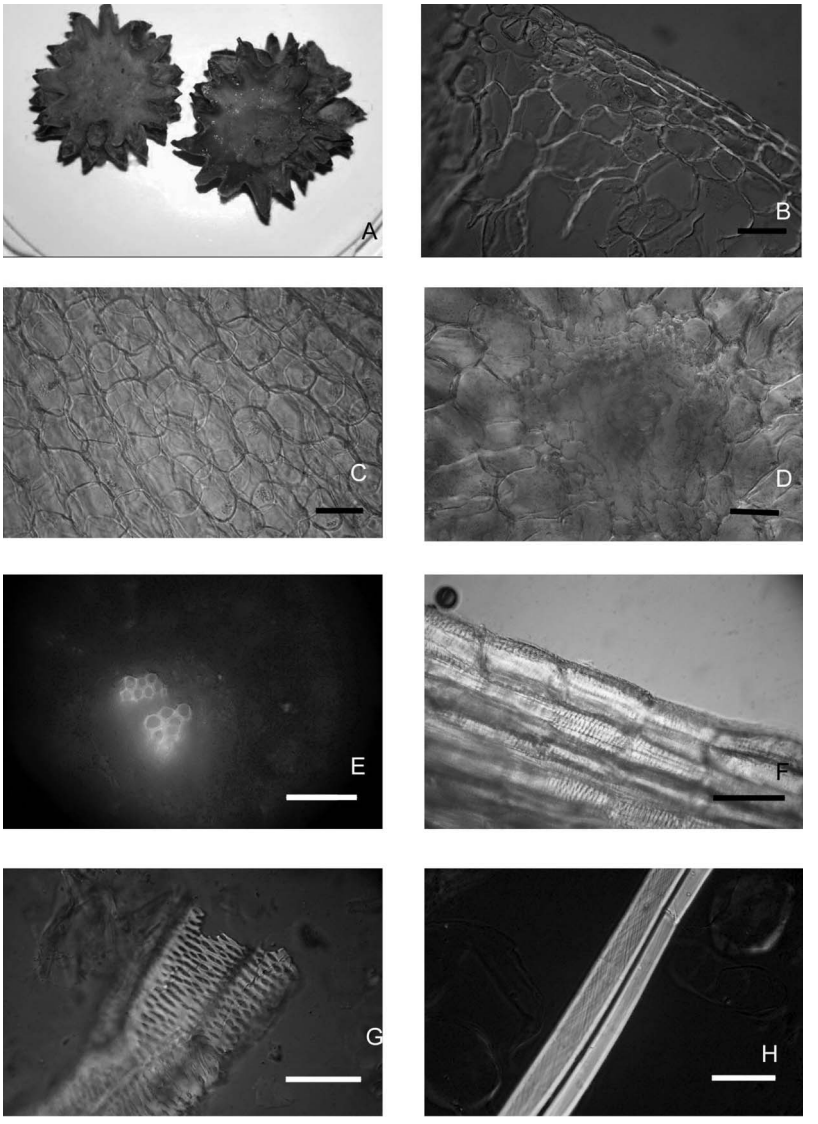

FIGURE 1 *H. gordonii*. (A) Macromorphology of *H. gordonii*; (B) transverse section showing epidermis, followed by single layer of hypodermis and cortex; (C) ovate-oblong cortical cells; (D) transverse section showing vascular bundles; (E) transverse section showing vessels under UV excitation 340 μm; (F) longitudinal section showing vessel with several joint cells; (G) vessels showing scalariform pitting; (H) fibers with simple horizontal striation (scale = 100 μm).

(Table 2). Of the 15 samples, 6 showed the presence of *Hoodia* plant material as evidenced by the observation of anatomical characteristics, such as presence of palisade cortical cells, fibers, vessels, and stomata that matched those of *Hoodia* spp. vouchers. These results confirmed that by using

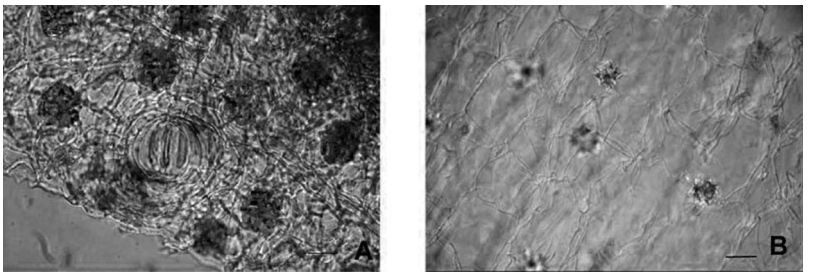

FIGURE 2 *O. ficus indica*. (A) Stomata and several oxalate crystals; (B) rectangular cortical cells with oxalate crystals (druses) (scale = 100 μm).

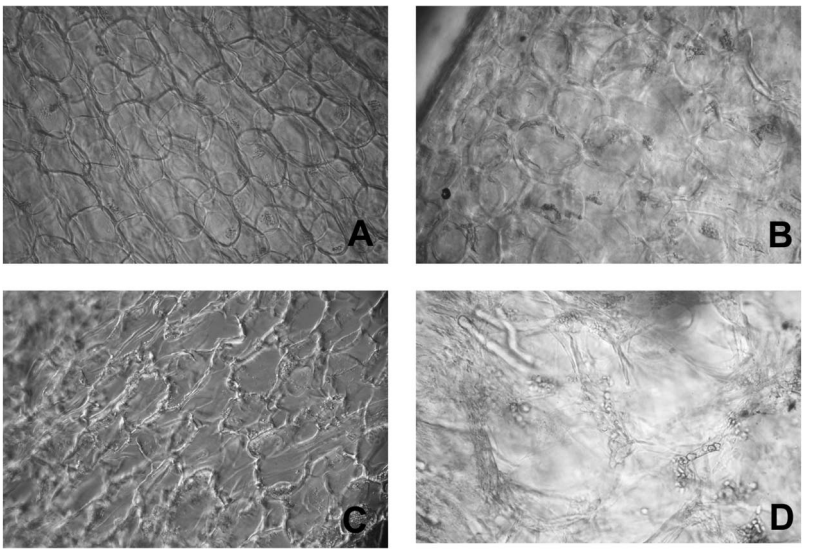

FIGURE 3 Various *Hoodia* spp. (A) Palisade cortical cells of *H. gordonii*; (B) palisade cortical cells of *H. currorii*; (C) palisade cortical cells of *H. ruschii* containing several starch grains; (D) *H. parviflora* showing palisade undulated, shrunken, collapsed cortical cells.

microscopy as a tool, the presence or absence of *Hoodia* plant material in commercially available dietary supplements could be confirmed.

Gene Amplification and Characterization

PCR products were obtained from all of the genomic DNAs when using the primers for *atp*B-*rbc*L and *psb*A-*trn*H intergenic regions. The sequence alignment of the *atp*B-*rbc*L intergenic region showed a relatively high homology (98%) between all of the analyzed *Hoodia* spp. samples and was not useful to design specific primers (data not shown). A significantly lower homology (65%) was observed between the *Hoodia* spp. and *Opuntia* spp.

and was suitable to design specific primers for *Opuntia* spp. The sequence alignment of the *psb*A-*trn*H intergenic region showed a 99% to 100% homology between *H. gordonii* samples 2925/2926/2927 (465bp) and the commercial samples 2887/2799/3126 (465bp). The *H. gordonii* samples showed a 97% to 98% homology to the *H. parviflora* samples 3162/3163 (474/475bp). The homology between the *H. gordonii* samples and *H. ruschii* samples 2822/2952 (420bp) was significantly lower at 89%. The lowest homology (62%) was observed between the *Hoodia* spp. samples and *Opuntia* spp. samples 2888/2886. Differences between the sequences of the analyzed species consisted of insertions, deletions, and nucleotide changes (data not shown). GenBank accession numbers of DNA sequences are as follows: FJ026605, FJ026606, FJ026607, FJ026608, FJ026609, FJ026610, FJ026611, FJ026612, FJ026613, FJ026614, FJ026615, FJ026616 FJ026617, and FJ026618.

Primer Design and Specificity Test

Based on the differences between the DNA sequences, three forward primers and two reverse primers were designed. For the nested PCR the *psb*A-*trn*H intergenic region was amplified first and the PCR product verified on an agarose gel (Fig. 4A). The designed primers were tested in single PCR reactions on the diluted *psb*A-*trn*H PCR products of the samples 2925, 2926, 2927, 2822, 2952, 2237, 2238, 2887, 2799, 3126, 2886, 2888, 2820, 2824, 2825, 2815, 2818, 2819, 2816, 2817, 2814, 2809, 2810, 2811, 2812, 2813 on ability to amplify fragments either from *Hoodia* spp., *Opuntia* spp., or other Asclepiadaceae samples. When the primer pair 1 was used, a PCR product with the expected size of about 90 bp was detected only from samples 2822 and 2952 (*H. ruschii*; Fig. 4B). The primer pair 2 resulted in a PCR product of the expected size of 137 bp of samples 3162, 3163 (*H. parviflora*) and of the expected size of 116 bp of samples 2925, 2926, 2927 (*H. gordonii*; Fig. 4B). The PCR product of the commercial samples 2887, 2799, 3126 was of the same size of the *H. gordonii* samples 2925, 2926, 2927. A PCR product with the expected size of about 145 bp was detected only from samples 2886, 2888 (*Opuntia* spp.) when primer pair 3 was used (Fig. 4C). No PCR product was detected from those *Opuntia* spp. samples with either primer pair 1 or 2. No PCR products from any of the other analyzed Asclepiadaceae species were detected with either primer pair 1, 2, or 3.

Genomic DNA from *Hoodia* tablets/capsules was extracted with various DNA extraction methods (not shown). Extracted DNA was subject to repeated PCR to amplify various genomic regions as done with genomic DNA from *Hoodia* plant samples. In contrast to genomic DNA isolated from plant samples, DNA isolated from tablets/capsules did not result in PCR products when applied to the same PCR master mix (Fig. 4).

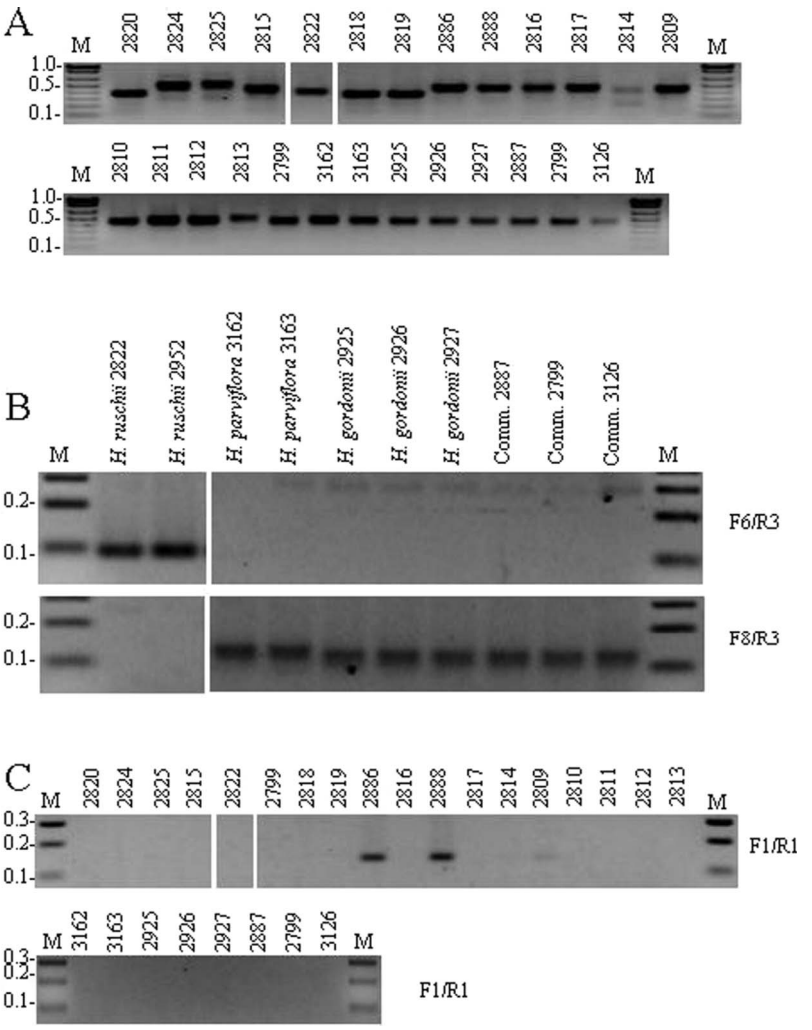

FIGURE 4 Agarose gel image of PCR products. (A) Using genomic DNA as the template and the primers for the *psb*A-*trn*H intergenic region a PCR product was detected from all the analyzed samples. (B) PCR products generated with primer pair 1 (F6/R3). A PCR product was only detected when DNA from samples 2822/2952 (*H. ruschii*) was present. The primer pair 2 (F8/R3) resulted in a PCR product of samples 3162/3163 (*H. parviflora*) and of samples 2925/2926/2927 (*H. gordonii*). The PCR product of the commercial samples 2887/2799/3126 was of the same size of the *H. gordonii* samples. (C) A PCR product was only detected when DNA from the *Opuntia* spp. and primer pair 3 (F1/R1) was used as template. M = molecular size standard, fragment sizes are given in kilo base pairs (kb).

CONCLUSION

Microscopy and molecular genetic procedures for the identification and differentiation between *H. gordonii*, *O. ficus indica*, and other related

Hoodia species were developed. Analyzed samples were dried plant material and processed plant material from commercially available tablets/capsules claiming to contain *Hoodia*. Using the provided detailed macro- and micromorphological descriptions enables easy validation of *H. gordonii* and other related species. The potential adulterant *O. ficus indica* could be easily distinguished from *Hoodia* spp. because the *O. ficus indica* showed the presence of a large oxalate crystal in cortical cells and had simple pitted vessels, unlike the *Hoodia* species. Microscopic analysis also allowed successful detection for the presence and absence of *Hoodia* plant material in the tested dietary supplements but could not definitively determine which *Hoodia* species was present (*H. gordonii*, *H. currorii*, *H. ruschii*, or *H. parviflora*).

The two intergenic regions *atp*B-*rbc*L and *psb*A-*trn*H were amplified and analyzed. These intergenic regions contain regions distinguishable between *H. gordonii*, *O. ficus indica*, and other related species such as *H. ruschii* and *H. parviflora*. Based on these results, primers were designed to amplify PCR products only when either *Hoodia* species or *O. ficus indica* DNA was present. Primers that could selectively identify *H. gordonii*, *H. parviflora*, and *H. ruschii* from other Hoodia species were also designed. Our results demonstrated that DNA identification and verification can be done by PCR from dried plant tissue. In contrast, no *Hoodia*-specific PCR product was amplified when extracted DNA from four different tablets/capsules was used as template. The absence of the PCR products is due to degraded DNA resulting from the manufacturing process of the tablets/capsules and limits the usefulness of our research when analyzing commercial products.

Authentication of medicinal plants is a critical issue, especially when the material is to be directly used as a medical treatment or as a botanical supplement. Harvesting, storage, and processing of botanicals can have a negative impact on the quality of the DNA, which can in some instances limit the use of molecular biology tools. A macroscopic/microscopic analysis can help to identify plant species but cannot identify marker compounds. A chemical analysis detects the presence of compounds but, in some instances, can be misleading if the samples are deliberately adulterated (spiked) with a marker compound. Unfortunately, no single superior method assures 100% authentication of botanicals. Several methodologies, such as macroscopic/microscopic analysis and identification based on DNA, as well as chemical profiling such as HPTLC or HPLC (2, 13), need to be applied in the correct manner to achieve the goal of identification and authentication of botanicals.

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